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| FORM PTO-139 (REV 1-96) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE | | ATTORNEY'S DOCKET NUMBER 3339-422 |
| TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371 | | U.S. APPLICATION NO. (If known, see 37 C.F.R. 1.5) 09/530560 |
| INTERNATIONAL APPLICATION NO PCT FR98/02344 | INTERNATIONAL FILING DATE November 3, 1998 | PRIORITY DATE CLAIMED November 3, 1997 |
| TITLE OF INVENTION TRANSFECTING PEPTIDE VECTOR, COMPOSITION CONTAINING SAME AND THEIR APPLICATIONS | | |
| APPLICANT(S) FOR DO/EO/US Jadwiga CHROBOCZEK; Pascal FENDER | | |
| Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information: | | |
| <ol style="list-style-type: none"> <input checked="" type="checkbox"/> This is a FIRST submission of items concerning a filing under 35 U.S.C. 371. <input type="checkbox"/> This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371. <input checked="" type="checkbox"/> This is an express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(l). <input type="checkbox"/> A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date. <input checked="" type="checkbox"/> A copy of the International Application as filed (35 U.S.C. 371(c)(2)) <ol style="list-style-type: none"> a. <input checked="" type="checkbox"/> is transmitted herewith (required only if not transmitted by the International Bureau). b. <input type="checkbox"/> has been transmitted by the International Bureau. c. <input type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US). <input checked="" type="checkbox"/> A translation of the International Application into English (35 U.S.C. 371(c)(2)). <input type="checkbox"/> Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3)) <ol style="list-style-type: none"> a. <input type="checkbox"/> are transmitted herewith (required only if not transmitted by the International Bureau). b. <input type="checkbox"/> have been transmitted by the International Bureau. c. <input type="checkbox"/> have not been made; however, the time limit for making such amendments has NOT expired. d. <input checked="" type="checkbox"/> have not been made and will not be made. <input type="checkbox"/> A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)). <input type="checkbox"/> An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)). <input type="checkbox"/> A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)). | | |
| Items 11. To 16. Below concern other document(s) or information included: | | |
| <ol style="list-style-type: none"> <input checked="" type="checkbox"/> An Information Disclosure Statement under 37 C.F.R. 1.97 and 1.98. <input type="checkbox"/> An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included. <input checked="" type="checkbox"/> A FIRST preliminary amendment. <input type="checkbox"/> A SECOND or SUBSEQUENT preliminary amendment. <input type="checkbox"/> A substitute specification. <input type="checkbox"/> A change of power of attorney and/or address letter. <input checked="" type="checkbox"/> Other items or information: Statement in Support of Filing a Sequence Listing, with descriptive printout; Labeled Computer Disk; and Copy of amendments made under Article 34 to the French PCT application. | | |

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| US APPLICATION NO 09/530560 | INTERNATIONAL APPLICATION NO PCT FR/98/02344 | ATTORNEY'S DOCKET NUMBER 3339-422 |
| 17. <input checked="" type="checkbox"/> The following fees are submitted: | | CALCULATIONS PTO USE ONLY |
| Basic National Fee (37 CFR 1.492(a)(1)-(5)): Neither international preliminary examination fee (37 CFR 1.482) nor International search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO | | \$970.00 |
| International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO | | \$840.00 |
| International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search (37 CFR 1.445(a)(2)) paid to USPTO | | \$690.00 |
| International preliminary examination fee (37 CFR 1.482) paid to USPTO But all claims did not satisfy provisions of PCT Article 33(1)-(4) | | \$670.00 |
| International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) | | \$ 96.00 |
| ENTER APPROPRIATE BASIC FEE AMOUNT | | = \$ 840.00 |
| SurchARGE of \$130.00 for furnishing the oath or declaration later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(e)). | | \$ |
| CLAIMS NUMBER FILED NUMBER EXTRA RATE | | |
| Total Claims | 17 -20 = 0 | X \$18.00 \$ 0.00 |
| Independent Claims | 3 - 3 = 0 | X \$78.00 \$ 0.00 |
| MULTIPLE DEPENDENT CLAIM(S) (if applicable) | | + \$260.00 \$ 260.00 |
| TOTAL OF ABOVE CALCULATIONS | | = \$ 1,100.00 |
| Reduction by ½ for filing by small entity, if applicable. A Small Entity statement must also be filed. (Note 37 CFR 1.9, 1.27, 1.28). | | \$ |
| SUBTOTAL | | = \$ |
| Processing fee of \$130.00 for furnishing the English translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(f)). | | \$ |
| TOTAL NATIONAL FEE | | = \$ 1,100.00 |
| Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40.00 per property + | | \$ |
| TOTAL FEES ENCLOSED | | = \$ 1,100.00 |
| | | Amount to be Refunded \$ |
| | | Charged \$ |
| a. <input checked="" type="checkbox"/> | A check in the amount of \$1,100.00 to cover the above fees is enclosed. | |
| b. <input type="checkbox"/> | Please charge my Deposit Account No. 16-0605 in the amount of \$ to cover the above fees. A duplicate copy of this sheet is enclosed. | |
| c. <input checked="" type="checkbox"/> | The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 16-0605. A duplicate copy of this sheet is enclosed. | |
| Note: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137 (a) or (b)) must be filed and granted to restore the application to pending status. | | |
| SEND ALL CORRESPONDENCE TO: Raymond Q. Linker, <i>Raymond Q. Linker</i> | | "Express Mail" Mailing Label Number EL 432837136 US Date of Deposit: May 2, 2000 |
| SIGNATURE REGISTRATION NUMBER 26,419 ALSTON & BIRD LLP Post Office Drawer 34009 Charlotte, NC 28234 Tel. Charlotte Office (704) 331-6000 Fax Charlotte Office (704) 334-2014 | | I hereby certify that this paper or fee is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above and is addressed to BOX PCT, Attn: DO/US (PTO) Assistant Commissioner for Patents, Washington, DC 20231. <i>Sarah B. Simmons</i> Sarah B. Simmons |
| Customer Number 000826 | | |

Attorney-Docket No. 3339-422

PATENT

IN THE UNITED STATES DESIGNATED OFFICE (DO/US)

In re: Chroboczek et al. Attn: DO/US
International Appl. No.: PCT/FR98/02344
International Filing Date: November 3, 1998
For: TRANSFECTING PEPTIDE VECTOR,
COMPOSITION CONTAINING SAME
AND THEIR APPLICATIONS

May 2, 2000

Box PCT
Assistant Commissioner of Patents
Washington, DC 20231

PRELIMINARY AMENDMENT

Sir:

Please amend the above-identified application as follows:

In The Specification:

Please add the heading --BACKGROUND OF THE INVENTION-- as Line 3 on Page 1 after the title of the application.

Please add the heading --SUMMARY OF THE INVENTION-- as Line 14 on Page 7 of the application before the paragraph beginning "The subject of...".

Please add the heading --BRIEF DESCRIPTION OF THE DRAWINGS-- as Line 9 on Page 13 of the application before the paragraph beginning "In addition to...".

Please add the heading DETAILED DESCRIPTION AND EXAMPLES as Line 7 on Page 14 of the application before the paragraph beginning "It should be...".

09520560-021500

In The Claims:

3. Transfection vector according to Claim 1 or Claim 2, [characterized in that] wherein the polymeric sequence of the basic amino acids comprises between 10 and 50 amino acid residues, selected from the group consisting of lysine, arginine and ornithine.
4. Transfection vector according to [any one of Claims 1 to 3, characterized in that] claim 1 or 2, wherein the cationic polymeric sequence is selected from the group consisting of polymeric amines.
5. Transfection vector according to [any one of Claims 1 to 4, characterized in that] claim 1 or 2, wherein the NLS sequence is at the N-terminal end of the transfecting peptide and the polymeric sequence of basic amino acids is at the C-terminal end of the said transfecting peptide.
6. Transfection vector according to [any one of Claims 1 to 5, characterized in that] claims 1 or 2, wherein, when the chemical substance is a nucleic acid, the transfecting peptide/nucleic acid ratio is between 0.3:1 and 15:1, preferably between 2:1 and 6:1 [, preferably between 4:1 and 6:1].
7. Transfection vector according to [any one of Claims 1 to 6, characterized in that it is] claims 1 or 2, combined with a targeting ligand.
8. [Composition, characterized in that it essentially consists] A composition consisting essentially of a transfection vector according to [any one of Claims 1 to 7] claim 1 or 2 and a suitable vehicle selected from the group consisting of bile salts, antiproteases, cyclodextrins and derivatives thereof, antiseptics and polyols [, for use as a medicament].
9. [Method] A method of transfecting eukaryotic cells *in vitro* with a chemical substance selected from the group consisting of nucleic acid sequences, proteins, peptides and

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pharmacologically active chemical substances, characterized in that it comprises the bringing into contact and the incubation of a transfection vector according to [any one of Claims 1 to 8,] claim 1 or 2 in a dilution buffer comprising 100 – 150 mM NaCl with eukaryotic cells for 15 to 120 minutes at room temperature, the chemical substance to be transfected:transfected peptide ratio being between 0.3:1 and 15:1, preferably between 2:1 and 6:1, preferably between 4:1 and 6:1.

10. Peptide vector for transfecting a chemical substance selected from the group consisting of nucleic acid sequences, proteins, peptides and pharmacologically active chemical substances, [characterized in that it contains,] containing, in addition to the said chemical substance, at least one transfecting peptide which comprises:

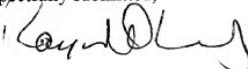
- a segment of an NLS sequence consisting of sequence ID NO:2,
- a segment of a sequence consisting of sequence ID NO:10,
- a segment of a sequence consisting of sequence ID NO:16, and
- a polylysine [, for use as a medicament].

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REMARKS

The above amendments are made to more clearly define the invention under United States practice. Please enter this amendment prior to calculation of the filing fee.

Respectfully submitted,



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CERTIFICATE OF EXPRESS MAILING

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Date of Deposit May 2, 2000

I hereby certify that this paper or fee is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above and is addressed to Box PCT, Assistant Commissioner For Patents, Washington, DC 20231.



Sarah B. Simmons

CLT01/4423464v1

TRANSPORTING PEPTIDE VECTOR, COMPOSITION CONTAINING
SAME AND THEIR APPLICATIONS

The present invention relates to a transfecting peptide vector, to a composition containing the said vector as well as to their applications in the treatment (medicaments) and the prevention (vaccines) of human and animal diseases. The said vector is in particular capable of dispensing to suitable target cells nucleic sequences, proteins, peptides and chemical substances of interest.

In the field of gene therapy, many compositions useful for efficiently transfecting eukaryotic cells with a selected genetic material have been described.

There are essentially two main types of transfection vectors:

- the natural transfection vectors, such as viruses or modified viruses, which are efficient but which have limits to their use: tissue nonspecificity,

necessity to obtain constructs for each gene of interest and potential risks for the environment which lead to the setting in place of costly and constraining clinical infrastructures for the patient and staff;

- nonviral agents (synthetic vectors), capable of promoting the transfer and the expression of chemical substances such as DNA into eukaryotic cells. The latter strategy represents an alternative to viral vectors.

These synthetic vectors must essentially have two functions: to condense the DNA to be transfected and to promote its cellular attachment as well as its passage across the plasma membrane and possibly the nuclear membranes; such vectors must therefore mimic the functioning of viruses in order to be efficient; however, it appears that the different vectors provided in the prior art do not exhibit these two functions in an optimum manner and may, in addition, depending on the cases, be toxic for the cells.

Among these nonviral agents, there may be mentioned first of all the cationic polymers and the cationic lipids. The former generally consist of polylysine, whereas a wide variety of cationic lipids (liposomes or pseudoliposomes) exist, each giving transfection efficiencies which vary according to the cell types (DOTMA, and the like).

The lipid portion which interacts with and/or destabilizes the membranes allows the fusion and the entry of the DNA/liposome complex.

However, the transfection of DNA by liposomes, although less immunogenic than that performed with the aid of cationic polymers, is in fact a relatively inefficient method.

The major mechanism of entry of the DNA/liposome complexes is, it seems, endocytosis; consequently, the transfected DNA is trapped in the intracellular vesicles and destroyed by the lysosomal enzymes.

Even if a portion of the transfected DNA is released into the cytoplasm by a mass action effect, only a small fraction of this DNA is effectively present in the nucleus.

Agents capable of increasing the release of the DNA from the endosomal vesicles and its passage into the nucleus can increase the gene transfer rate.

Among these agents, there may be distinguished:
- those which target the complex towards another point of entry: the targeting is obtained, for example, by coupling ligands with the polylysine polymers; the targeting can also occur after internalization, by directing the complexes to the nucleus (PCT International Application WO 95/31557), and

- those which avoid endosomal degradation; to escape endosomal degradation, it has been proposed to incorporate an endosomolytic agent into the complex, such as adenoviral particles (PCT International

Application WO 93/07283) or more recently synthetic peptides with endosomolytic activity, which increase the release of the DNA into the cytoplasm.

Taking into account the preceding text, various 5 types of complexes have been provided; there may be mentioned complexes combining liposomes and peptides, such as those described in:

- International Application WO 96/25508, which describes compositions comprising (i) the nucleic acid 10 to be transfected, (ii) a transfection agent, such as a cationic polymer and/or a lipofectant, (iii) a peptide compound involved at the level of the condensation of the nucleic acid, consisting as a whole or in part of peptide motifs possessing a majority of amino acids 15 with a basic character, such as lysine, histidine, arginine (histones, nucleolin, protamine or derivatives thereof) and optionally (iv) a targeting element which makes it possible to orient the transfer of the nucleic acid, such as a ligand of the intracellular type such 20 as a nuclear localization signal sequence (NLS) which favours the accumulation of the transfected DNA inside the nucleus and which may be combined with the peptide compound to form a chimeric peptide comprising a protein fragment (histone or protamine or nucleolin) 25 and an NLS sequence. However, this system requires the presence of a cationic polymer and/or a lipofectant, which have the disadvantage of being toxic and/or costly,

- International Application WO 97/30170 which 30 also describes compositions for transfecting eukaryotic cells, which comprise the nucleic acid to be transfected, at least one cationic lipid at a suboptimal concentration and at least one acidic peptide (active on the membrane) which destabilizes the 35 endosomal membrane and thus increases the transfection efficiency. The positive charge/negative charge ratio is between 0 and 3. The selected peptides are derived from the influenza virus, so as to induce effective

rupture of the endosomes. The presence of the lipids is necessary in this composition because of the fact that the selected peptide does not allow passage across the first cell membrane.

5 Such complexes do not therefore make it possible to avoid the disadvantages linked to the use of liposomes.

That is undoubtedly the reason why complexes using only peptides have been provided:

10 - European Patent Application 0,544,292, which describes a complex for transfecting nucleic acid which comprises a fusion protein consisting of a cellular factor (growth factor, viral antigen, toxin, integrin or lipoprotein) and a basic polycationic peptide comprising arginine and/or lysine residues,

15 - International Application WO 94/23751, which describes a transfer peptide which comprises three parts: (1) a ligand L1 (peptide of 2 to 100 amino acids), capable of binding to a binding site at the surface of eukaryotic cells (membrane receptor) (example: peptide RGD, domain for binding of growth factors, hormones, viral antigens or lipoproteins), (2) a ligand L2 similar to L1 (peptide of 2 to 20 amino acids), which binds to the outer nuclear membrane of eukaryotic cells, such as an NLS sequence and (3) a ligand L3 corresponding to a basic peptide (3 to 100 amino acids) (histone fragment H1 or H2B, for example). The transfer peptides described in this Application therefore have a general ligand structure for a membrane receptor-ligand for the outer nuclear membrane-basic peptide. Such a structure was proposed in order to improve the specificity of the complex towards target cells, but has a toxicity level of the same order as that of the liposomes; in addition, the 20 construct should be adapted as a function of the target cells (presence of specific receptors on the target cells), and

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International Application WO 95/31557 which describes a transfection vector comprising a synthetic peptide and the nucleic acid to be transfected. The synthetic peptide comprises a polymeric chain of basic amino acids, preferably at the C-terminal position (10-50 amino acids, such as lysine, arginine and ornithine), an NLS peptide (6-15 amino acids, such as the NLS sequence of the SV40 T antigen, the NLS sequence of adenovirus Ela or the NLS sequence of adenovirus Elb, preferably at the N-terminal position and a hinge region of neutral amino acids (6-50 amino acids selected from glycine, alanine, leucine and isoleucine), between the polymeric chain and the NLS peptide. The preferred NLS sequence is the sequence of the SV40 virus T antigen (small sequence of basic amino acids: PKKKRKV), which is efficient in mammalian cells or a short hydrophobic sequence which contains one or more basic amino acids (KIPIK). The hinge sequence comprises 6-26 neutral amino acids selected solely from Gly (G), Ala (A), Leu (L) and Ile (I). The peptide:DNA ratio (by weight) is between 1:1 and 1:10. The peptide described in this document passes across the cell membrane with difficulty and that is the reason why it is recommended, in this International Application, to treat the cells before the transfection: the cells are treated with a hypertonic solution, and then with a hypotonic solution in the presence of the nucleic acid-peptide complex. The hypertonic solution may contain PEG (0.3 M-0.6 M) and sucrose (10-25%).

These various complexes possess the property of condensing the DNA and of promoting its combination with the cell membrane; however, they are far from being as efficient as the viral vectors, in particular because of an insufficient condensation of the DNA to be transfected and/or of the difficulties encountered by the transfected DNA in coming out of the endosome

without being degraded and in penetrating into the cell nucleus.

Seeking to develop novel vectors not exhibiting the disadvantages of the viral vectors, the inventors 5 developed the vector described in International Application WO 97/18317, which describes compositions comprising an adenoviral protein complex consisting:

A. of an adenoviral protein complex, namely:

- either of 12 pentons, each comprising at 10 least one fibre and one penton base, excluding any other constituent element of the genome of an adenovirus, which fibre(s) and penton base are derived either from the same adenovirus, or from different adenoviruses, the said pentons being linked by the 15 penton bases and forming a dodecahedron structure, stable to proteolytic enzymes, which complex has a molecular weight of between 4.8×10^6 and 6.6×10^6 ;

- or of 12 penton bases, excluding any other constituent element of the genome of an adenovirus, 20 which penton bases are derived either from the same adenovirus, or from different adenoviruses, and form a dodecahedron structure, stable to proteolytic enzymes and in that it has a molecular weight of between 3.2×10^6 and 4×10^6 .

25 B. of a nucleic acid sequence to be transfected, and

C. of a ligand between the adenoviral protein complex and the nucleic acid, such as peptides whose N-terminal portion comprises the N-terminal amino acid 30 sequence of a fibre of an adenovirus of any serotype (region for attachment to the adenoviral protein complex) and whose C-terminal portion comprises a polylysine or a polyarginine.

The transfection vectors described in this 35 Application allow the internalization of the nucleic sequence to be transfected and increase the permeability of the endosomes; this is, however, a relatively complex structure which mimics the behaviour

of adenoviruses; indeed, the adenoviral particles are relatively complex and comprise several substructures; in particular the outer part or capsid is formed predominantly of three proteins: the hexon, the penton base and the fibre; the fibre allows the attachment of the virion to a cell receptor, whereas the penton base allows the internalization of the virion.

Continuing their research studies, the inventors have found that, unexpectedly, a peptide derived from the adenovirus fibre protein is capable of efficiently transfecting nucleic acid sequences or proteins, in the absence of liposomes and of the treatment of cells.

The subject of the present invention is a peptide vector for transfecting a chemical substance selected from the group consisting of nucleic acid sequences, proteins, peptides and pharmacologically active chemical substances, characterized in that it contains, in addition to the said chemical substance, at least one transfecting peptide derived from the whole or part of an adenovirus fibre and comprising at least one region consisting of at least 50% of hydrophobic amino acids selected from the group consisting of alanine, valine, phenylalanine, isoleucine, leucine, proline and methionine.

In accordance with the invention, the said peptide is derived from a fibre of an adenovirus selected from the group consisting of Ad2, Ad3, Ad4, Ad7, Ad8, Ad9, Ad11, Ad12, Ad15, Ad16, Ad21, Ad40, Ad41, FAV1 (CELO) and FAV7 or from a fragment of the SV40 virus Vp3 protein.

According to an advantageous embodiment of the said transfection vector, the said transfecting peptide comprises at least:

- a segment of an NLS sequence derived from an adenovirus fibre comprising between 4 and 5 amino acids and including a sequence selected from the group consisting of the following sequences: X₀-Lys-Arg-Val-

- Arg (X_0 KRVR) (SEQ ID NO:1), X_0 -Lys-Arg-Ala-Arg (X_0 KRAR) (SEQ ID NO:2), X_0 -Lys-Arg-Ser-Arg (X_0 KRSR) (SEQ ID NO:3), X_0 -Lys-Arg-Leu-Arg (X_0 KRLR) (SEQ ID NO:4), X_0 -Lys-Arg-Thr-Arg (X_0 KRTR) (SEQ ID NO:5), X_0 -Pro-Lys-Lys-Pro-Arg (X_0 PKKPR) (SEQ ID NO:6), in which X_0 is zero or represents Thr (T), Ala (A), Ser-Lys (SK) or Ser (S), or a segment of the SV40 virus Vp3 protein and in particular the sequence GPNKKKRKL (SEQ ID NO:24),
5 - a hydrophobic sequence comprising between 7 and 50 amino acids, derived from an adenovirus fibre and selected from the group consisting of at least one of the following sequences X_1 -Phe-Asn-Pro-Val-Tyr-Pro-Tyr- X_2 (X_1 FNPVYPYX $_2$) (SEQ ID NO:7), X_1 -Phe-Asp-Pro-Val-Tyr-Pro-Tyr- X_2 (X_1 FDPVYPYX $_2$) (SEQ ID NO:8), in which:
10 15 X_1 is zero or represents a sequence of at most 43 amino acids, preferably a sequence of 5 to 15 amino acids, comprising hydrophobic and/or polar and/or acidic charged amino acids, and in particular one of the following sequences: Leu-Ser-Asp-Ser (LSDS) (SEQ ID NO:9), Leu-Ser-Thr-Ser (LSTS) (SEQ ID NO:10), Leu-Ser-Ser-Ser (LSSS) (SEQ ID NO:11), Pro-Ser-Glu-Asp-Thr (PSEDT) (SEQ ID NO:12), Val-Asp-Asp-Gly (VDDG) (SEQ ID NO:13), Thr-Gln-Tyr-Ala-Glu-Glu-Thr-Glu-Glu-Asn-Asp-Asp (TQYAEETEENDD) (SEQ ID NO:14) or X_1 -Glu-Asp-Asp (X_1 EDD) (SEQ ID NO:15) in which X_1 represents Ala (A), Val (V), Leu (L), Phe (F) or Ile (I) and
20 25 X_2 is zero or represents a sequence of at most 43 amino acids, preferably a sequence of 5 to 15 amino acids, comprising hydrophobic and/or polar and/or charged amino acids, and in particular one of the following sequences: Glu-Asp-Glu-Ser (EDES) (SEQ ID NO:16), Asp-Thr-Glu-Thr (DTET) (SEQ ID NO:17), Asp-Ala-Asp-Asn (DADN) (SEQ ID NO:18), Asp-Pro-Phe-Asp (DPFD) (SEQ ID NO:19), Gly-Tyr-Ala-Arg (GYAR) (SEQ ID NO:20),
30 35 Glu-His-Tyr-Asn (EHYN) (SEQ ID NO:21), Asp-Thr-Ser-Ser (DTSS) (SEQ ID NO:22) or Asp-Thr-Phe-Ser (DTFS) (SEQ ID NO:23) and

- a polymeric sequence of basic amino acids or a cationic polymeric sequence or a polyalcohol.

There are understood by:

- hydrophobic amino acids, the following amino acids: Ala, Val, Leu, Ile, Pro, Phe and Met;
 - acidic charged amino acids, the following amino acids: Asp and Glu;
 - basic charged amino acids, the following amino acids: Lys, Arg and ornithine; and
 - neutral polar amino acids, the following amino acids: Gly, Ser, Thr, Cys, Tyr, Asn, Gln, His and Trp.

Advantageously, the said transfecting peptide is branched and comprises at least two fragments which are derived from an adenovirus fibre; the said fragments each comprise a segment of an NLS sequence and a hydrophobic sequence, as defined above and are linked to each other by a polymeric sequence such as a polymeric sequence of basic amino acids.

20 When the chemical substance is a nucleic acid sequence, it is selected from genes which encode a polypeptide having a therapeutic activity, antisense sequences and ribozymes.

In the case of a coding sequence, it comprises,
25 in addition, an active promoter for the expression of
the polypeptide.

The said promoter is in particular selected from the group consisting of constitutive promoters and inducible promoters.

Surprisingly, such a transfecting peptide vector comprising no lipids (in the form of liposomes, for example) or penton base is capable of efficiently transfecting in particular nucleic acid sequences of any size, up to the nucleus and without poisoning the transfected cell.

In all cases, the exogenous nucleic acid sequence, the protein of interest or any other chemical

substance, combined with the said transfection vector penetrates into the cell (internalization).

Surprisingly, the transfecting peptide-cell receptor interaction significantly increases both the internalization of the transfection vector and the permeability of the endosomes, which significantly increases the passage of the exogenous nucleic acid, of the protein of interest or of any other chemical substance from the endosomes to the cytoplasm and to the nucleus, in comparison with the use of a vector including lipids (in the form of liposomes, for example).

Such transfecting peptide vectors prove surprisingly more efficient and less toxic than compositions containing liposomes (cationic lipids or lipofectants).

According to another advantageous embodiment of the said transfection vector, the said transfecting peptide comprises at least:

- a segment of an NLS sequence derived from an adenovirus fibre comprising between 4 and 5 amino acids and including a sequence selected from the group consisting of the following sequences: X_0 -Lys-Arg-Val-Arg (X_0 KRVR) (SEQ ID NO:1), X_0 -Lys-Arg-Ala-Arg (X_0 KRAR) (SEQ ID NO:2), X_0 -Lys-Arg-Ser-Arg (X_0 KRSR) (SEQ ID NO:3), X_0 -Lys-Arg-Leu-Arg (X_0 KRLR) (SEQ ID NO:4), X_0 -Lys-Arg-Thr-Arg (X_0 KRTT) (SEQ ID NO:5), X_0 -Pro-Lys-Lys-Pro-Arg (X_0 PKKPR) (SEQ ID NO:6), in which X_0 is zero or represents Thr (T), Ala (A), Ser-Lys (SK) or Ser (S), or a segment of the SV40 virus Vp3 protein and in particular the sequence GPNKKKRKL (SEQ ID NO:24),

- a hydrophobic sequence comprising between 7 and 50 amino acids, derived from an adenovirus fibre and selected from the group consisting of at least one of the following sequences X_1 -Phe-Asn-Pro-Val-Tyr-Pro-Tyr- X_2 (X_1 FNPVVPYX $_2$) (SEQ ID NO:7), X_1 -Phe-Asp-Pro-Val-Tyr-Pro-Tyr- X_2 (X_1 FDPVYPYX $_2$) (SEQ ID NO:8), in which:

X₁ is zero or represents a sequence of at most 43 amino acids, preferably a sequence of 5 to 15 amino acids, comprising hydrophobic and/or polar and/or acidic charged amino acids, and in particular one of 5 the following sequences: Leu-Ser-Asp-Ser (LSDS) (SEQ ID NO:9), Leu-Ser-Thr-Ser (LSTS) (SEQ ID NO:10), Leu-Ser-Ser-Ser (LSSS) (SEQ ID NO:11), Pro-Ser-Glu-Asp-Thr (PSEDT) (SEQ ID NO:12), Val-Asp-Asp-Gly (VDDG) (SEQ ID NO:13), Thr-Gln-Tyr-Ala-Glu-Glu-Thr-Glu-Glu-Asn-Asp-Asp 10 (TQYAEETEENDD) (SEQ ID NO:14) or X₁-Glu-Asp-Asp (X₁,EDD) (SEQ ID NO:15) in which X₁ represents Ala (A), Val (V), Leu (L), Phe (F) or Ile (I) and

X₂ is zero or represents a sequence of at most 43 amino acids, preferably a sequence of 5 to 15 amino acids, comprising hydrophobic and/or polar and/or charged amino acids, and in particular one of the 15 following sequences: Glu-Asp-Glu-Ser (EDES) (SEQ ID NO:16), Asp-Thr-Glu-Thr (DTET) (SEQ ID NO:17), Asp-Ala-Asp-Asn (DADDN) (SEQ ID NO:18), Asp-Pro-Phe-Asp (DPFD) 20 (SEQ ID NO:19), Gly-Tyr-Ala-Arg (GYAR) (SEQ ID NO:20), Glu-His-Tyr-Asn (EHYN) (SEQ ID NO:21), Asp-Thr-Ser-Ser (DTSS) (SEQ ID NO:22) or Asp-Thr-Phe-Ser (DTFS) (SEQ ID NO:23), which transfecting peptide is combined with a polymeric sequence of basic amino acids, a cationic 25 polymer or a polyalcohol.

According to another advantageous embodiment of the said transfecting peptide vector, the polymeric sequence of basic amino acids comprises between 10 and 50 amino acid residues, selected from the group 30 consisting of lysine, arginine and ornithine.

According to another advantageous embodiment of the said transfecting peptide vector, the cationic polymeric sequence is selected from the group consisting of polyamines and quaternary ammonium 35 polymers; a preferred polyamine is polyethyleneimine (PEI).

In accordance with the invention, the said polyalcohol is preferably a C₃-C₂₀, and in particular glycerol or dextrans.

According to another advantageous embodiment of
5 the said transfecting peptide vector, the NLS sequence
is at the N-terminal end of the transfecting peptide
and the polymeric sequence of basic amino acids is at
the C-terminal end of the said transfecting peptide.

According to another advantageous embodiment of
10 the said transfecting peptide vector, when the chemical
substance is a nucleic acid, the transfecting peptide/nucleic acid ratio is between 0.3:1 and 15:1,
preferably between 2:1 and 6:1, preferably between 4:1
and 6:1.

15 According to another advantageous embodiment of
the said transfecting peptide vector, it is combined
with a targeting ligand.

The subject of the invention is also a
composition, characterized in that it essentially
20 consists of a transfer vector as defined above and a
suitable vehicle selected from the group consisting of
bile salts, antiproteases, cyclodextrins and
derivatives thereof, antiseptics and polyols.

The compositions according to the invention
25 have many applications as medicaments, in human and
veterinary medicine:

- in human and animal gene therapy, in particular in hereditary diseases,
- as antiviral agents (antisense sequences or
30 ribozymes),
- as immunogenic or vaccinal agents,
- as antibacterial or anticancer agents, and the like.

The subject of the present invention is also a
35 method of transfecting eukaryotic cells *in vitro* with a
chemical substance selected from the group consisting
of nucleic acid sequences, proteins, peptides and
pharmacologically active chemical substances,

characterized in that it comprises the bringing into contact and the incubation of a transfecting peptide vector in accordance with the invention, in a dilution buffer comprising 100-150 mM NaCl with eukaryotic cells
5 for 15 to 120 minutes at room temperature, the chemical substance to be transfected:transflecting peptide ratio being between 0.3:1 and 15:1, preferably between 2:1 and 6:1, preferably between 4:1 and 6:1.

In addition to the preceding features, the
10 invention also comprises other features which will emerge from the description which follows, which refers to exemplary embodiments of the method which is the subject of the present invention as well as to the accompanying drawings, in which:

15 - Figure 1 illustrates the transfer of the luciferase gene with peptide I, as a function of time (Figure 1A) or as a function of the NaCl concentration (Figure 1B);

20 - Figure 2 illustrates the kinetics of the expression of the luciferase gene; this figure comprises on the x-axis the transfecting peptide/DNA or DOTAP/DNA ratios and on the y-axis the percentage of transfection at D1 (■), D2 (□), D3 (□) and D6 (□);

25 - Figure 3 represents the migrations obtained on a retardation gel, as a function of the quantity of transfecting peptide;

30 - Figure 4 illustrates the transfections obtained with the liposomes DOSPER and DOTAP; this figure comprises on the x-axis the liposome/DNA ratios and on the y-axis the RLU (Relative Light Unit)/10 sec/10⁵ cells;

35 - Figure 5 illustrates the kinetics of entry into the cells of a peptide (peptide I) observed in confocal microscopy; column A shows the fluorescent peptide and column B shows the cellular nucleic acids stained with propidium iodide;

- Figure 6 illustrates the inhibition of transfection with peptide I, after preincubation with

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an excess of peptide I. The HeLa cells in plates comprising 24 wells are preincubated with peptide I for one hour at 4°C at concentrations of 10 to 50 µg/ml, respectively;

5 - Figure 7 represents a few sequences of adenovirus fibres.

It should be clearly understood, however, that these examples are given solely by way of illustration of the subject of the invention and do not constitute
10 in any manner a limitation thereof.

EXAMPLE 1: Materials and methods

- Cells, plasmid and peptides:

The HeLa cells are cultured at 37°C in an EMEM
15 medium supplemented with 10% foetal calf serum under an atmosphere containing 5% CO₂.

A luciferase reporter vector (plasmid pGL3, Promega) is used to demonstrate the transfection.

The peptide IC comprises sequence No. 2,
20 sequence No. 10, sequence No. 7, sequence No. 16, from the N-terminal end to the C-terminal end and 10 lysines; this peptide corresponds to the 20 N-terminal residues of the Ad3 fibre. Peptide I contains these same 20 N-terminal amino acids of the Ad3 fibre and 20
25 lysines; the peptides were obtained by solid-phase synthesis, followed by HPLC purification.

This peptide I is labelled with fluorescein; peptides comprising 10 lysines instead of 20 were also prepared. The integrity of all the peptides is verified
30 by mass spectroscopy.

- Retardation gel:

500 ng of plasmid DNA (pGL3) are preincubated with various quantities of peptides for 5 min at room temperature, and then subjected to electrophoresis on a
35 1% agarose gel prepared in a TBE buffer.

After electrophoresis at 50 V in the TBE buffer for 30 min, the DNA on the gel being stained with

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ethidium bromide and visualized under ultraviolet light.

- Transfections:

5 1.5 to 12 µg of peptide I in 50 µl of dilution buffer (20 mM Tris, pH 7.4, 150 mM NaCl) are incubated with 1.5 µg of plasmid pGL3 in 250 µl of EMEM medium for 15 to 30 min at room temperature.

10 The mixture of DOTAP or of DOSPER with 1.5 µg of plasmid pGL3 is prepared according to the manufacturer's instructions (Boehringer). For the studies of the effect of the peptide on transfection in the presence of liposomes, portions of peptides are mixed with DOTAP or DOSPER in the dilution buffer defined above for 15 to 30 min at room temperature, and
15 1.5 µg of plasmid pGL3 are added and incubated for 15 min at room temperature.

The transfections are carried out in plates comprising 24 wells (Beckton Dickinson) with 1.5×10^3 cells/well (confluence of about 50%) for 1 h at 37°C.
20 After 24 h, the light emission is measured in the cell lysates with the Promega Luciferase Assay System test.

- Haemolytic test:

25 Human erythrocytes are washed 3 times with PBS buffer. 10^6 erythrocytes are incubated with 6 µg of peptide I for various periods of time at 37°C. After centrifugation at 10,000 g for 5 min, the optical density of the supernatant is measured at 540 nm.

- Internalization of the peptide:

30 HeLa cells, cultured on cover slips (10^5 cells/cover slip) are treated with 3% bovine serum albumin in EMEM for 15 min at 37°C.

35 The cells are washed twice with PBS buffer, incubated with 40 µg/ml of peptide I labelled with fluorescein for various durations at 37°C, fixed with 2% paraformaldehyde in PBS for 20 min at 37°C, washed with PBS and stained with 1 µg/ml of propidium iodide in PBS for 5 min at room temperature.

The cover slips are mounted on a microscope slide with 1,4-diazabicyclooctane (Sigma) and observed under an MRC600 confocal microscope (BioRad).

* Results:

5 - Cell transfection with peptide I:

All the experiments were carried out with HeLa cells transfected with the plasmid pGL3 (Stratagene) carrying the luciferase gene. For peptide I/DNA ratios equal to 2, the optimum time for interaction between 10 the transfecting complexes and the cells is between 60 and 120 min (Figure 1A).

The effect of the NaCl concentrations was tested for 1 h of transfection on gene expression measured 24 h after transfection (Figure 1B).

15 A transfection optimum exists for peptide I/DNA ratios of between 4 and 6 and for an NaCl concentration of 125 mM. Concentrations of less than 100 mM and greater than 150 mM appear to be inhibitory.

20 The expression of the transgene may be observed up to 6 days after transfection (Figure 2). However, the addition of 2% serum completely abolishes the transfection with peptide I.

25 The behaviour of the DNA/peptide complexes is analysed on retardation gels. In theory, 526 peptide molecules are necessary to neutralize the phosphate charges carried by the plasmid (5256 bp), which means that 322 ng of peptide I are necessary for complete neutralization of 500 ng of plasmid.

30 Figure 3 shows that incubation with 250 ng of peptide causes retardation in the migration of the DNA and the addition of 500 ng of peptide completely stops its migration, which is in agreement with the theoretical considerations set out above.

35 The highest transfection efficiency is observed when an excess of neutralizing peptide charges relative to the DNA of the order of 4 exists (Figure 1B), which confirms that the transfer of the gene occurs only in the presence of an excess of positive charges.

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- Parameters involved in the efficiency of transfection with the peptide according to the invention:

The peptides according to the invention 5 comprise essentially 3 domains: the nuclear localization signal, the hydrophobic domain and the basic polymer.

To study the effect of the structure of the peptide on the transfection of DNA, a series of 10 peptides [lacuna], in which the various portions of peptide I have been removed, whose sequences are illustrated in Table I below:

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TABLE I

| Peptide | Sequences* |
|---------|---|
| I | A K R A R L S T S F N P V Y P Y E D E S - K ₂₀ = SEQ ID NO:2 + SEQ ID NO:10 + SEQ ID NO:7 + SEQ ID NO:16-K ₂₀ |
| IC | A K R A R L S T S F N P V Y P Y E D E S - K ₁₀ = SEQ ID NO:2 + SEQ ID NO:10 + SEQ ID NO:7 + SEQ ID NO:16-K ₁₀ |
| IE | A K R A R L S T S E D E S-K ₁₀ = SEQ ID NO:2 + SEQ ID NO:10 + SEQ ID NO:16- K ₁₀ |
| ID | L S T S F N P V Y P Y E D E S-K ₂₀ = SEQ ID NO:10 + SEQ ID NO:7 + SEQ ID NO:16- K ₂₀ |
| IA | A K R A R L S T S F N P V Y P Y E D E S = SEQ ID NO:2 + SEQ ID NO:10 + SEQ ID NO:7 + SEQ ID NO:16 |
| LII | A K R A R L S T S F N P V Y P Y E D E S A K R A R L S T S F N P V Y P Y E D E S (for each branch: SEQ ID NO:2 + SEQ ID NO:10 + SEQ ID NO:7 + SEQ ID NO:16) |

* in which X₀ = A.

5 The results obtained with these various peptides are illustrated in Table II below.

TABLE II

| Peptide (μ g) | DNA (μ g) | RLU/10 s/ 10^5 cells $\times 10^3$ |
|-------------------------------|----------------|--------------------------------------|
| <u>Peptide I</u> | | |
| 3 | 1.5 | 450 |
| 6 | 1.5 | 4920 |
| 9 | 1.5 | 1450 |
| 12 | 1.5 | 1170 |
| <u>Peptide IC</u> | | |
| 3.6 | 1.5 | 1 |
| 9 | 1.5 | 2870 |
| 12 | 1.5 | 1330 |
| <u>Peptide IE</u> | | |
| 3 | 1.5 | 1 |
| 6 | 1.5 | 10 |
| 9 | 1.5 | 110 |
| 12 | 1.5 | 330 |
| <u>Peptide ID</u> | | |
| 3-12 | 1.5 | 1 |
| <u>Peptide IA</u> | | |
| 3-12 | 1.5 | 1 |
| <u>Peptide K₁₀</u> | | |
| 3-12 | 1.5 | 1 |
| <u>Peptide K₂₀</u> | | |
| 3-12 | 1.5 | 1-10 |

The results presented in Table II show that the
 5 transfection efficiency depends on the presence of the
 nuclear localization signal sequence of the adenovirus
 fibre protein.

There is no transfection when the NLS domain
 (peptide ID), polylysine (peptide IA) or both the NLS
 10 domain and the hydrophobic region (peptide K₁₀ and
 peptide K₂₀) are removed. The removal of the hydrophobic
 domain between the NLS portion and the polylysine
 portion induces a significant effect: this peptide
 (peptide IE) is approximately 30 times less efficient
 15 than peptide IC. Even if peptide ID is still capable of

attaching and condensing the DNA and of entering into the cell, it appears nevertheless incapable of transporting this DNA into the nucleus.

It is also evident from these results that when
5 the number of lysines present in the polylysine polymer (peptide IC) is reduced, a modest negative effect is observed on the transfection efficiency which can be compensated for by increasing the quantity of peptide necessary for an efficient transfer.

10 Table III below illustrates other results obtained with peptide I or peptide IA, in the presence of glycerol or of PEI and peptide LII.

TABLE III

| Peptide (or PEI) (μ g) | DNA (μ g) | RLU/10 s/ 10^5 cells $\times 10^3$ |
|-----------------------------|----------------|--------------------------------------|
| <u>Peptide I</u> | | |
| 6 | 1.5 | 12,600 |
| 6 (+ 10% glycerol) | 1.5 | 34,900 |
| 6 (+ 0.1% DMSO) | 1.5 | 11,320 |
| <u>Peptide LII</u> | | |
| 1.5 | 1.5 | 0 |
| 3 | 1.5 | 5200 |
| 6 | 1.5 | 2400 |
| <u>PEI</u> | | |
| 3 | 1.5 | 66,400 |
| 4 | 1.5 | 51,200 |
| <u>Peptide IA + PEI</u> | | |
| 1.2 μ g | 1.5 | 104,000 |
| 1.6 μ g | 1.5 | 84,800 |

The peptide vector comprising peptide I and PEI (covalent complex) is more efficient than PEI (polyethyleneimine) alone.

20 The transfection efficiency is significantly greater in the case of the covalent complex.

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It can be said that 1 µg of peptide I/PEI gives 54,000-67,000 RLU, while 1 µg of PEI gives 13,000-22,000 RLU.

5 Effect of the peptide on transfection in the presence of liposomes:

Two liposomes were used, the liposome DOTAP and the liposome DOSPER, both marketed by Boehringer.

They consist of cationic lipids with internal ester bonds capable of being degraded by cellular esterases or esterases, which ought to confer on these liposomes a cytotoxicity lower than that observed with other liposomes.

The conditions for transfecting the HeLa cells were optimized for the liposomes alone (Figure 4). The

15 transfection with the liposome DOTAP leads to lower efficiencies than those observed with the liposome DOSPER; however, the transfections with the liposome DOTAP tend to show a plateau above a certain DNA/liposome ratio, whereas the transfections with the
20 liposome DOSPER show a peak. For the simultaneous peptide/liposome transfections, the order in which these compounds are added is important, since a higher efficiency (increase of several factors) is observed when the liposome is first mixed with the peptide (and
25 not with the DNA) and then when the plasmid DNA is added after 15 min of incubation at room temperature.

Table IV below illustrates the results obtained.

TABLE IV

| | | RLU/10 sec/ 10^6 cells $\times 10^3$ | | Peptide I:DNA | |
|-------------------------|------------------|--|-------------------|-------------------|--|
| DOTAP:DNA no peptide I | | 2 | | 4 | |
| 2 | 9320 \pm 820 | 28,900 \pm 890 | | 30,440 \pm 2360 | |
| 4 | 18,590 \pm 570 | 15,050 \pm 340 | | 17,750 \pm 750 | |
| 6 | 4140 \pm 640 | 3800 \pm 770 | | 7300 \pm 150 | |
| DOSPER:DNA no peptide I | | Peptide I:DNA | | Peptide I:DNA | |
| 4 | 822 \pm 78 | 1 | 2 | 4 | |
| 5.5 | 6880 | 3160 \pm 760 | 11,102 \pm 2370 | 5140 | |
| | | 18,820 | 13,760 | 12,840 | |
| No liposome | | 13,550 \pm 2930 | | 35,500 \pm 4800 | |

The results illustrated in this Table IV were obtained under the following conditions:

portions of the peptide I are mixed with 1.5 µg of plasmid pGL3 in 300 µl of dilution buffer for 15 min at room temperature.

The liposome DOTAP is mixed with 1.5 µg of plasmid pGL3 in a DOTAP/DNA ratio of 2 or 4 (v/w), in 300 µl of dilution buffer for 15 min at room temperature.

Surprisingly, these results show that in the absence of liposomes, the results observed with the peptide alone in peptide/DNA ratios of 4 are superior to those observed with the liposome/peptide mixture.

- Intracellular location of the peptide:

The cellular distribution of the fluorescent peptide I is monitored by confocal microscopy (Figure 5).

The first observations, carried out 2 min after the transfection, show a certain quantity of signal at the periphery of the cell.

5 to 10 min after the addition of the peptide, a strong cytoplasmic signal is observed, indicating the entry of the peptide into the cell.

At 30 min, the signal is also observed in the nucleus and a very bright signal is observed in the nucleoli. This substantial transfer into the nucleoli is particularly surprising.

The observations made between 60 and 120 min show the passage of the peptide again into the cytoplasm and at the periphery of the cell until the signal is completely lost.

The results illustrated in this Figure 4 were obtained with peptide concentrations twice as high (40 µg/ml) as those used for the DNA transfection assays. When the usual concentrations for the transfections are used, the accumulation of peptides is slower, but follows the same steps as those specified above.

- Mechanism of the internalization of the peptide:

A haemolytic test carried out with peptide I on erythrocytes gives negative results which show that the interaction of the cell with peptide I is not linked to the formation of pores. Since no expression of luciferase is observed when the transfections are carried out at 4°C, it appears that the mechanism of internalization of the peptide depends on endocytosis and involves the cytoskeleton.

To determine if sites for specific attachment of peptide I to the plasma membrane exist, cells were preincubated with peptide I at 4°C for 2 h, so as to try to saturate the possible sites of attachment of the peptide.

This resulted in complete inhibition of transfection (Figure 6), which indicates that the latter involves a receptor-dependent endocytosis.

- Size of the transfecting complex:

Light scattering can be used to measure the size of the complexes and their distribution, if a mixed population is formed. Under these conditions, it is possible to study the effect of the incubation time on the formation of the transfecting complexes.

The size is measured immediately after the mixing and 1 h later. The complexes prepared by mixing the liposome DOTAP with a DNA plasmid have a diameter of about 115 nm and their size does not change during the incubation.

The DNA/peptide I complexes are larger and have a diameter of about 350-360 nm. Furthermore, the formation of the complex is a dynamic process since a rapid increase in size is observed as a function of the incubation time when more than 90% of the complexes reach a diameter of 660-1100 nm after 1 h.

The size and the distribution of the complexes with peptide I is similar regardless of the peptide/DNA ratio (from 1 to 8).

The effect of the size of the complex on the transfection efficiency was studied using complexes prepared by varying the period of incubation at room temperature. The results are illustrated in Table V
5 below.

TABLE V

RLU/10 sec/10⁵ cells x 10³

| Peptide:DNA | Preincubation time at room temperature (min) | | |
|----------------------|--|--------|--------|
| <u>Experiment 1:</u> | 15 | 60 | 120 |
| 2 | 410 | 150 | 827 |
| 4 | 2540 | 1900 | 1460 |
| 6 | 702 | 3460 | 980 |
| <u>Experiment 2:</u> | 0 | 15 | 30 |
| 4 | 400 | 3240 | 4950 |
| <u>Experiment 3:</u> | 0 | 15 | 30 |
| 4 | 1710 | 14,550 | 23,170 |
| | | 32,175 | 6800 |

10 Surprisingly, with the complexes according to the invention, even very large aggregates can be transfected.

15 To measure the size of the transfected substance, light scattering measurements were carried out with an ion-argon laser (Spectra Physics 1161) at 488 nm and 150 mW (geometry of scattering at 90°). The spectrum is accumulated for 200 s using a Malvern 7032 correlator (Malvern Instruments) and then repeated 1 h later. All the spectra are in homodyne mode: the 20 amplitudes of the intensity correlation function with a zero retardation are consistent with the special coherence factor β obtained with a suspension of dilute latex, namely $\beta = 0.90$.

25 The hydrodynamic rays R_h are calculated using the Malvern multimodal procedure (Pike-Ostrowsky), in order to characterize the principal rates of degradation of the field correlation function with the Stokes-Einstein equation $R_h = k_b T Q^2 / (6\pi\eta\Gamma_1)$, in which Γ_1

is the principal degradation rate, T is the absolute temperature of the thermal bath (298 K), Q the transfer wave vector and η the viscosity of the solvent.

As is evident from the above, the invention is
5 not at all limited to its embodiments, methods of implementation and methods of application which have just been described more explicitly; it embraces, on the contrary, all the variants which may occur to a specialist in this field, without departing from the
10 framework or scope of the present invention.

CLAIMS

1. Peptide vector for transfecting a chemical substance selected from the group consisting of nucleic acid sequences, proteins, peptides and pharmacologically active chemical substances, characterized in that it contains, in addition to the said chemical substance, at least one transfecting peptide derived from the whole or part of a fibre of an adenovirus selected from the group consisting of Ad2, Ad3, Ad4, Ad7, Ad8, Ad9, Ad11, Ad12, Ad15, Ad16, Ad21, Ad40, Ad41, FAV1 (CELO) and FAV7, which transfecting peptide comprises at least:

15 - a segment of an NLS sequence derived from an adenovirus fibre comprising between 4 and 5 amino acids and including a sequence selected from the group consisting of the following sequences: X_0 -Lys-Arg-Val-Arg (X_0 KRVR) (SEQ ID NO:1), X_0 -Lys-Arg-Ala-Arg (X_0 KRAR) (SEQ ID NO:2), X_0 -Lys-Arg-Ser-Arg (X_0 KRSR) (SEQ ID NO:3), X_0 -Lys-Arg-Leu-Arg (X_0 KRLR) (SEQ ID NO:4), X_0 -Lys-Arg-Thr-Arg (X_0 KRTA) (SEQ ID NO:5), X_0 -Pro-Lys-Lys-Pro-Arg (X_0 PKKPR) (SEQ ID NO:6), in which X_0 is zero or represents Thr (T), Ala (A), Ser-Lys (SK) or Ser (S), or a segment of the SV 40 virus Vp3 protein and in particular the sequence GPNKKKRKL (SEQ ID NO:24),

25 - a hydrophobic sequence comprising between 7 and 50 amino acids, derived from an adenovirus fibre and selected from the group consisting of at least one of the following sequences X_1 -Phe-Asn-Pro-Val-Tyr-Pro-Tyr- X_2 (X_1 FNPVYPYX $_2$) (SEQ ID NO:7), X_1 -Phe-Asp-Pro-Val-Tyr-Pro-Tyr- X_2 (X_1 FDPVYPYX $_2$) (SEQ ID NO:8), in which:

30 X_1 is zero or represents a sequence of at most 43 amino acids, preferably a sequence of 5 to 15 amino acids, comprising hydrophobic and/or polar and/or acidic charged amino acids, and in particular one of the following sequences: Leu-Ser-Asp-Ser (LSDS) (SEQ ID NO:9), Leu-Ser-Thr-Ser (LSTS) (SEQ ID NO:10), Leu-Ser-Ser-Ser (LSSS) (SEQ ID NO:11), Pro-Ser-Glu-Asp-Thr

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(PSEDT) (SEQ ID NO:12), Val-Asp-Asp-Gly (VDDG) (SEQ ID NO:13), Thr-Gln-Tyr-Ala-Glu-Glu-Thr-Glu-Glu-Asn-Asp-Asp (TQYAEETEENDD) (SEQ ID NO:14) or X₁-Glu-Asp-Asp (X₁EDD) (SEQ ID NO:15) in which X₁ represents Ala (A), Val (V),

5 Leu (L), Phe (F) or Ile (I) and

X₂ is zero or represents a sequence of at most 43 amino acids, preferably a sequence of 5 to 15 amino acids, comprising hydrophobic and/or polar and/or charged amino acids, and in particular one of the
10 following sequences: Glu-Asp-Glu-Ser (EDES) (SEQ ID NO:16), Asp-Thr-Glu-Thr (DTET) (SEQ ID NO:17), Asp-Ala-Asp-Asn (DADN) (SEQ ID NO:18), Asp-Pro-Phe-Asp (DPFD) (SEQ ID NO:19), Gly-Tyr-Ala-Arg (GYAR) (SEQ ID NO:20), Glu-His-Tyr-Asn (EHYN) (SEQ ID NO:21), Asp-Thr-Ser-Ser
15 (DTSS) (SEQ ID NO:22) or Asp-Thr-Phe-Ser (DTFS) (SEQ ID NO:23) and

- a polymeric sequence of basic amino acids or a cationic polymeric sequence or a polyalcohol,
for use as a medicament.

20 2. Peptide vector for transfecting a chemical substance selected from the group consisting of nucleic acid sequences, proteins, peptides and pharmacologically active chemical substances, characterized in that it contains, in addition to the
25 said chemical substance, at least one transfecting peptide derived from the whole or part of a fibre of an adenovirus selected from the group consisting of Ad2, Ad3, Ad4, Ad7, Ad8, Ad9, Ad11, Ad12, Ad15, Ad16, Ad21, Ad40, Ad41, FAV1 (CELO) and FAV7, which transfecting peptide comprises at least:

- a segment of an NLS sequence derived from an adenovirus fibre comprising between 4 and 5 amino acids and including a sequence selected from the group consisting of the following sequences: X₀-Lys-Arg-Val-
35 Arg (X₀KRVR) (SEQ ID NO:1), X₀-Lys-Arg-Ala-Arg (X₀KRAR) (SEQ ID NO:2), X₀-Lys-Arg-Ser-Arg (X₀KRSR) (SEQ ID NO:3), X₀-Lys-Arg-Leu-Arg (X₀KRLR) (SEQ ID NO:4), X₀-Lys-Arg-Thr-Arg (X₀KRTR) (SEQ ID NO:5), X₀-Pro-Lys-Lys-

Pro-Arg (X_0 PKKPR) (SEQ ID NO:6), in which X_0 is zero or represents Thr (T), Ala (A), Ser-Lys (SK) or Ser (S), or a segment of the SV40 virus Vp3 protein and in particular the sequence GPNKKKRKL (SEQ ID NO:24),

5 - a hydrophobic sequence comprising between 7 and 50 amino acids, derived from an adenovirus fibre and selected from the group consisting of at least one of the following sequences X_1 -Phe-Asn-Pro-Val-Tyr-Pro-Tyr- X_2 (X_1 FNPVYPYX $_2$) (SEQ ID NO:7), X_1 -Phe-Asp-Pro-Val-Tyr-Pro-Tyr- X_2 (X_1 FDPVYPYX $_2$) (SEQ ID NO:8), in which:

10 X_1 is zero or represents a sequence of at most 43 amino acids, preferably a sequence of 5 to 15 amino acids, comprising hydrophobic and/or polar and/or acidic charged amino acids, and in particular one of the following sequences: Leu-Ser-Asp-Ser (LSDS) (SEQ ID NO:9), Leu-Ser-Thr-Ser (LSTS) (SEQ ID NO:10), Leu-Ser-Ser-Ser (LSSS) (SEQ ID NO:11), Pro-Ser-Glu-Asp-Thr (PSEDT) (SEQ ID NO:12), Val-Asp-Asp-Gly (VDDG) (SEQ ID NO:13), Thr-Gln-Tyr-Ala-Glu-Thr-Glu-Glu-Asn-Asp-Asp (TQYAEETEENND) (SEQ ID NO:14) or X_1 -Glu-Asp-Asp (X_1 EDD) (SEQ ID NO:15) in which X_1 represents Ala (A), Val (V), Leu (L), Phe (F) or Ile (I) and

15 X_2 is zero or represents a sequence of at most 43 amino acids, preferably a sequence of 5 to 15 amino acids, comprising hydrophobic and/or polar and/or charged amino acids, and in particular one of the following sequences: Glu-Asp-Glu-Ser (EDES) (SEQ ID NO:16), Asp-Thr-Glu-Thr (DTET) (SEQ ID NO:17), Asp-Ala-Asp-Asn (DADN) (SEQ ID NO:18), Asp-Pro-Phe-Asp (DPFD) (SEQ ID NO:19), Gly-Tyr-Ala-Arg (GYAR) (SEQ ID NO:20), Glu-His-Tyr-Asn (EHYN) (SEQ ID NO:21), Asp-Thr-Ser-Ser (DTSS) (SEQ ID NO:22) or Asp-Thr-Phe-Ser (DTFS) (SEQ ID NO:23), which transfecting peptide is combined with a polymeric sequence of basic amino acids, a cationic polymer or a polyalcohol, for use as a medicament.

20 3. Transfection vector according to Claim 1 or Claim 2, characterized in that the polymeric sequence

of basic amino acids comprises between 10 and 50 amino acid residues, selected from the group consisting of lysine, arginine and ornithine.

4. Transfection vector according to any one of
5 Claims 1 to 3, characterized in that the cationic polymeric sequence is selected from the group consisting of polymeric amines.

5. Transfection vector according to any one of
10 Claims 1 to 4, characterized in that the NLS sequence is at the N-terminal end of the transfecting peptide and the polymeric sequence of basic amino acids is at the C-terminal end of the said transfecting peptide.

10. Transfection vector according to any one of
15 Claims 1 to 5, characterized in that when the chemical substance is a nucleic acid, the transfecting peptide/nucleic acid ratio is between 0.3:1 and 15:1, preferably between 2:1 and 6:1, preferably between 4:1 and 6:1.

20. Transfection vector according to any one of
20 Claims 1 to 6, characterized in that it is combined with a targeting ligand.

25. Composition, characterized in that it essentially consists of a transfection vector according to any one of Claims 1 to 7 and a suitable vehicle selected from the group consisting of bile salts, antiproteases, cyclodextrins and derivatives thereof, antiseptics and polyols, for use as a medicament.

30. Method of transfecting eukaryotic cells *in vitro* with a chemical substance selected from the group consisting of nucleic acid sequences, proteins, peptides and pharmacologically active chemical substances, characterized in that it comprises the bringing into contact and the incubation of a transfection vector according to any one of Claims 1 to 8, in a dilution buffer comprising 100-150 mM NaCl with eukaryotic cells for 15 to 120 minutes at room temperature, the chemical substance to be transfected:transfected peptide ratio being between

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0.3:1 and 15:1, preferably between 2:1 and 6:1,
preferably between 4:1 and 6:1.

10. Peptide vector for transfecting a chemical substance selected from the group consisting of nucleic acid sequences, proteins, peptides and pharmacologically active chemical substances, characterized in that it contains, in addition to the said chemical substance, at least one transfecting peptide which comprises:

- 10 - a segment of an NLS sequence consisting of sequence ID NO:2,
 - a segment of a sequence consisting of sequence ID NO:10,
 - a segment of a sequence consisting of sequence ID NO:16, and
 - a polylysine,
for use as a medicament.

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LEGEND TO THE FIGURES

Legend to Figure 1

1. Time (min)
- 5 2. Peptide:DNA ratio

Legend to Figure 2

- 10 1. Percentage of transfection
2. Peptide/DNA
3. DOTAP/DNA
4. Day

15 Legend to Figure 4

1. RLU/10 sec/ 10^5 cells
2. Liposome/DNA ratio

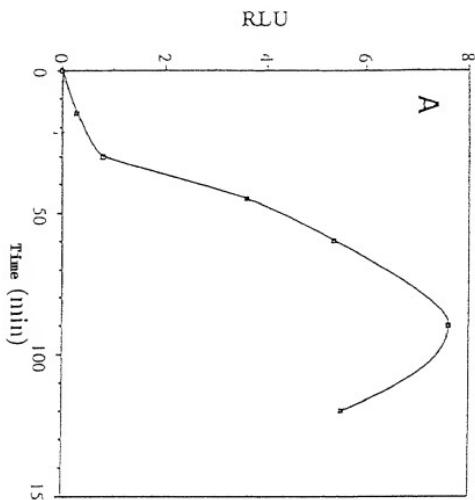
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Legend to Figure 6

1. Peptide I/DNA ratio
2. Control

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Peptide



Peptide/NaCl

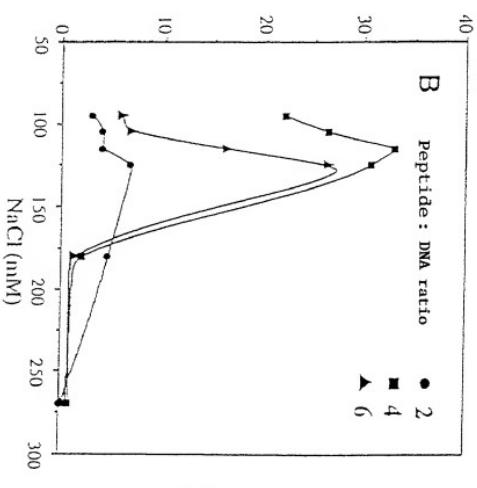


FIGURE 1

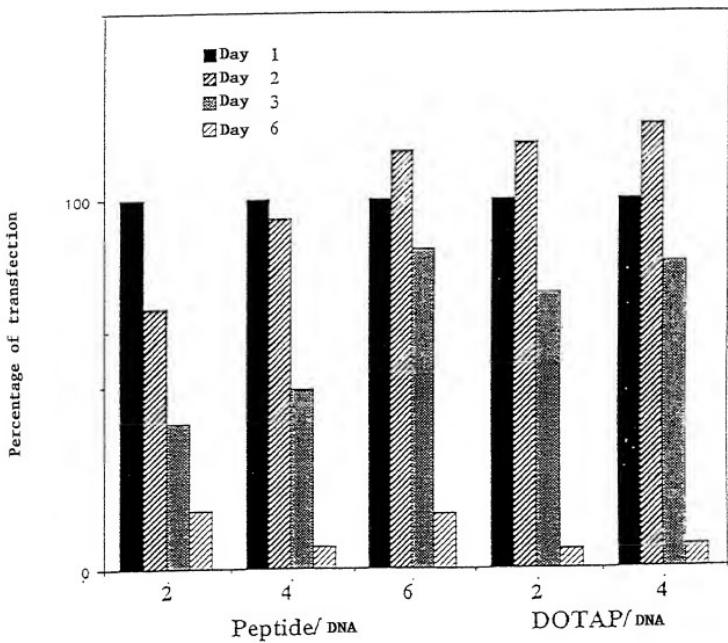


FIGURE 2

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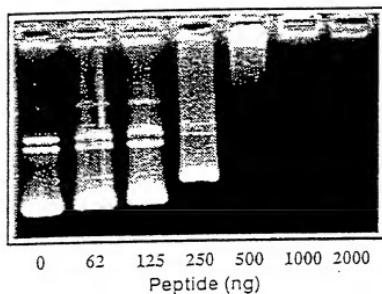
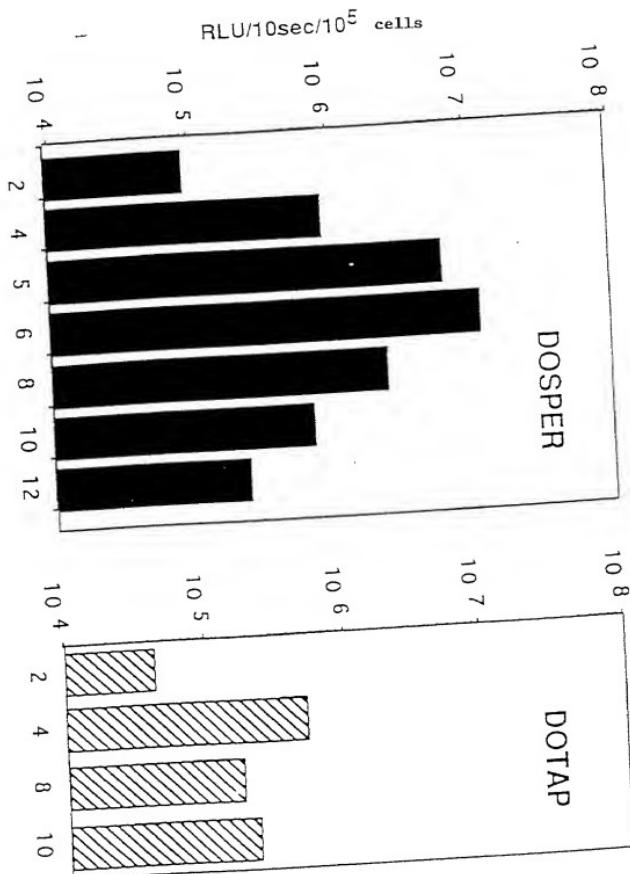


FIGURE 3



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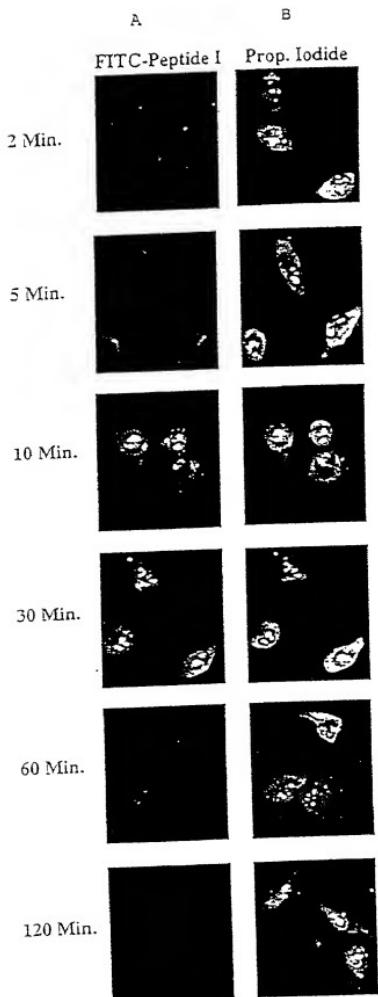
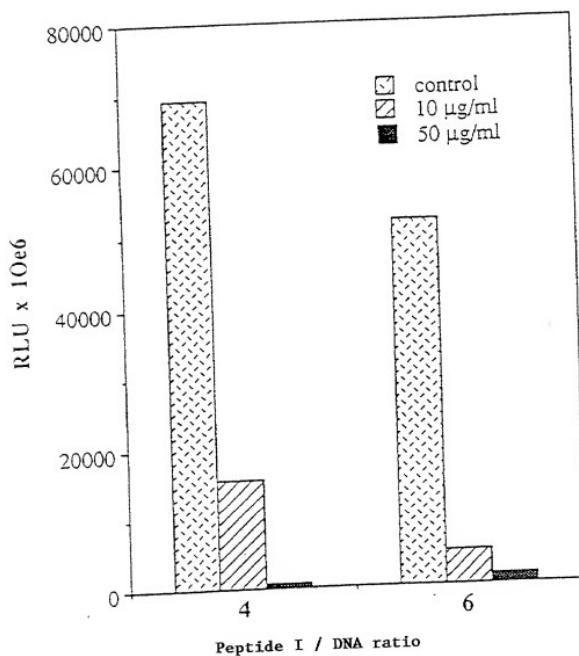


FIGURE 5

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FIGURE 6

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| | | | |
|--------|----------------------------|--------------------------|-----------|
| AD1p | WT - KRAL S - - - - | DSFNPVYPYEDESTSQ - - | HPF1NPGF |
| AD11a | WT - KRAL S - - - - | DSFNPVYPYEDESTSQ - - | HPF1NPGF |
| AD7 | WT - KRAL S - - - - | DSFNPVYPYEDESTSQ - - | HPF1NPGF |
| AD21 | WT - KRAL S - - - - | DSFNPVYPYEDESTSQ - - | HPF1NPGF |
| 1 | MA - <u>KRAT1S</u> - - - - | TSFNPVYPYEDESSQ - - | HPF1NPGF |
| AD16 | MA - KRAL S - - - - | SSFNPVYPYEDESSQ - - | HPF1NPGF |
| AD2 | M - KRAP S - - - - | EDTFNPVYPYDTEGPP - - | VPFELTPPF |
| AD5 | M - KRAP S - - - - | EDTFNPVYPYDTEGPP - - | VPFELTPPF |
| AD4 | M SKK KRAL R - - - - | DGFDPVYPYDADNA PT - | VPF1NPPF |
| AD12 | M - KRSRTOQAYEETEEEND | DPFDTSO - - VPFVTPPF | |
| AD6 | WT - KRL R - - - - | EDDFNPVYPGYARNON - - | IPLFLTPPF |
| AD9 | MS - KRL R - - - - | EDDFNPVYPGYARNON - - | IPLFLTPPF |
| AD15 | MS - KRL R - - - - | EDDFNPVYPGYARNON - - | IPLFLTPPF |
| AD41-1 | M - <u>KRAL R</u> - - - - | EDDFNPVYPYEHYN - PLD - I | PFLITTPPF |
| AD40-1 | M - KRAL R - - - - | EDDFNPVYPYEHYN - PLD - I | PFLITTPPF |
| AD40-2 | M - <u>KTRI</u> - - - - | EDDFNPVYPYOTSS - TPS - I | PYVAPPF |
| AD41-2 | M - KTRI - - - - | EDDFNPVYPYOTSS - TPS - I | PYVAPPF |

FIGURE 7

DECLARATION AND POWER OF ATTORNEY FOR PATENT APPLICATION

(Foreign Agent Involved)

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name. I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled
Transfecting peptide vector, composition containing same and their applications

the specification of which is attached hereto unless the following box is checked:

[] was filed on _____ as United States Application Number or PCT International Application Number _____ and was amended on _____ (applicable).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR § 1.56

I hereby claim foreign priority benefits under 35 U.S.C. § 119(a)-(d) or § 365(b) of any foreign application(s) for patent or inventor's certificate, or § 365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or PCT international application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application(s)

Priority Claimed

| | | | | |
|----------------|-----------|------------------------|-------|-----|
| PCT/FR98/02344 | FRANCE | 3 November 1998 | [X] | [] |
| (Number) | (Country) | (Day/Month/Year Filed) | Yes | No |
| 97 13771 | FRANCE | 3 November 1997 | [X] | [] |
| (Number) | (Country) | (Day/Month/Year Filed) | Yes | No |

I hereby claim the benefits under 35 U.S.C. § 119(e) of any United States provisional application(s) listed below.

| | |
|----------|---------------|
| (Number) | (Filing Date) |
| (Number) | (Filing Date) |

I hereby claim the benefit under 35 U.S.C. § 120 of any United States application(s), or § 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C., § 112, I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR § 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application.

| | | |
|---------------------|---------------|----------------------------------|
| (Appln. Serial No.) | (Filing Date) | (Status --patented/pending/abn.) |
|---------------------|---------------|----------------------------------|

| | | |
|---------------------|---------------|----------------------------------|
| (Appln. Serial No.) | (Filing Date) | (Status --patented/pending/abn.) |
|---------------------|---------------|----------------------------------|

The undersigned hereby authorizes the U.S. attorney or agent named herein to accept and follow instructions from my French representatives, Cabinet Ores, as to any action to be taken in the U.S. Patent and Trademark Office regarding this application without direct communication between the U.S. attorney or agent and the undersigned. In the event of a change in the persons from whom instructions may be taken, the U.S. attorney or agent named herein will be so notified by the undersigned.

I hereby appoint the practitioners associated with the Customer Number provided below to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith, and direct that all correspondence be addressed to that Customer Number:

Customer Number 826

| | |
|---|---|
| ADDRESS CORRESPONDENCE TO THE ATTENTION OF: Raymond O. Linker, Jr. Registration No. 26,419 | DIRECT ALL TELEPHONE CALLS TO: Raymond O. Linker, Jr. Registration No. 26,419 Tel (704) 331-6000 Fax (704) 334-2014 |
|---|---|

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

1 - 80

Full name of first/sole inventor: Jadwiga CHROBOCZEK

Inventor's Signature: Jadwiga Chrobocek Date: 4/24/2000

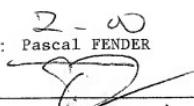
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Full name of second inventor: Pascal FENDER

Inventor's Signature: 

Date: 26/04/2000

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Citizenship: French

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Pfx

Full name of third inventor:

Inventor's Signature: _____

Date: _____

Residence:

Citizenship:

Post Office Address:

DRAFTS & DESIGNS

Full name of fourth inventor:

Inventor's Signature: _____

Date: _____

Residence:

Citizenship:

Post Office Address:

159780

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526 Rec'd PCT/PTO 02 MAY 2000

Attorney's Docket No. 3339-422

PATENT

IN THE UNITED STATES DESIGNATED OFFICE (DO/US)

In re: Chroboczek et al.
Appl. No.: PCT/FR98/02344
Filed: November 3, 1998
For: TRANSFECTING PEPTIDE VECTOR,
COMPOSITION CONTAINING SAME
AND THEIR APPLICATIONS

Attn: DO/US

May 2, 2000

**STATEMENT IN SUPPORT OF FILING A
SEQUENCE LISTING UNDER 37 CFR § 1.821(f)**

Box PCT
Assistant Commissioner for Patents
Washington, DC 20231

Sir:

I hereby state that the content of the paper and computer readable copies of the Sequence Listing, submitted concurrently herewith in accordance with 37 CFR § 1.821(c) and (e), are the same.

Respectfully submitted,



Raymond O. Linker, Jr.
Attorney/Agent for Applicant
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"Express Mail" Mailing Label Number EL 432837136 US
Date of Deposit: May 2, 2000

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Sarah B. Simmons

CERTIFICATE OF MAILING

I hereby certify that this correspondence is being deposited with the United States Postal Service with sufficient postage as first class mail in an envelope addressed to: Assistant Commissioner For Patents, Washington, DC 20231, on May 1, 2000.

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526 Rec'd PGYPTO 02 MAY 2000

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FENDER, Pascal
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CENTRE NATIONAL DE LA RECHERCHE SCIENTIFIQUE-CNRS

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DRAFT - 03/2009